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Growth and development of the knapweed root weevil, *Cyphocleonus achates*, on a meridic larval diet

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Abstract

Cyphocleonus achates, the knapweed weevil, is an effective biological control agent of the invasive weed, *Centaurea maculosa* Lam. A meridic diet was developed and tested for the rearing of the larval stage of this insect. Using this diet, *C. achates* was reared for over three generations, with the adults being offered knapweed plants for feeding and oviposition in greenhouse conditions. Slight or no differences were seen between insects reared on a standard meridic diet formulation and one containing knapweed tissues. The following life history parameters were monitored over the three generations: percent egg hatch (ranging from 42.9 to 59.1%), time to egg hatch (20.0–23.2 days), time to adult emergence (52.0–54.1 days), adult weights 3 days post-eclosion (101.9–117.0 mg), percent adult emergence (48.3–58.6%), and percent mortality/deformity in the different stages (with mortality occurring primarily in the early larval stages). Additionally, a study involving low temperature and short day conditions suggested that *C. achates* could be maintained for longer periods of time in larval diet cells when placed in growth-retarding conditions, although percent adult emergence was lower. External morphology was also studied in order to distinguish between the sexes to ensure that each adult cage had a similar ratio of females to males. Abdominal features were found to be the most dependable characteristics for use when determining the sex of adult *C. achates*.
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1. Introduction

Spotted knapweed, *Centaurea maculosa* Lam. (Asteraceae), is a highly invasive, noxious plant that was introduced into the United States in the 1800s and has infested rangelands, pastures, forests, and roadsides throughout the United States and Canada (Duncan, 2001; Gleason and Cronquist, 1991; Harris and Myers, 1984; Sheley et al., 1998). The mechanisms by which *Ce. maculosa* out-competes native plants are not completely understood but include enhanced resource allocation and allelopathy (Bais et al., 2003; LeJeune and Seasted, 2001; Ridenaur and Callaway, 2001), with the former being aided by knapweed's interaction with arbuscular mycorrhizal fungi (Carey et al., 2004). A variety of control measures are available to combat *Ce. maculosa*, which include biological control agents (both insects and pathogens; Caesar et al., 2002; Lang et al., 2000; Sheley et al., 1998), alteration of nutrient availability (Herron et al., 2001), and herbicides (Jacobs et al., 2000; Sheley et al., 1998). One insect used to control spotted knapweed is the knapweed root weevil, *Cyphocleonus*

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achates (Fahraeus) (Coleoptera: Curculionidae) (Clark et al., 2001; Lang et al., 2000; Stinson et al., 1994; Story et al., 1997; Wikeem and Powell, 1999). However, low fecundity and variable developmental times, as well as an inability to obtain adults year round, can limit their use both geographically and seasonally.

In the field, *C. achates* are univoltine with adults emerging over a 2- to 3-month period in late summer to mid-fall, with the exact months depending upon the geographical location (Clark et al., 2001; Stinson et al., 1994; Story et al., 1997; Wikeem and Powell, 1999). Adults feed on knapweed leaves and oviposit eggs in the root or root crown. The resulting larvae feed on and pupate in the root (Stinson et al., 1994).

We obtained field populations of *C. achates* adults and reared them under a combination of laboratory and greenhouse conditions, using both a meridic diet (i.e., a diet for which the chemical identity of most of the essential molecules are known) for feeding and development in the larval stage, and host plants for feeding and oviposition in the adult stage. Our primary objective was to determine if our meridic diet (with or without knapweed tissue) would generate reproductive adults over a number of generations. We found that, unlike other diets developed for weevils, especially root-boring curculionids (Alarcón et al., 2002; Blossey et al., 2000; Blossey and Eberts, 2001; Farinos et al., 1999; Hunt et al., 1992; Shimoji and Yamagishi, 2004; Thomas, 1969; Trudel et al., 1994; Zerillo and Odell, 1972), our diet produced insects with stable rearing times, survivorship, and fecundity over three generations without the addition of host tissue. Furthermore, we determined that we could store developing larvae in our diet over a short time-period. Additionally, we developed a method for sexing adults to ensure that the ovipositional cages contained similar sex ratios.

2. Materials and methods

2.1. Evaluation of insect growth, development, and reproduction

In the fall of 2001, adults reared in corrals in Montana (Story et al., 1996) were shipped to BCIRL (courtesy of L. White and J. Story, Department of Entomology, Montana State University, Bozeman, MT; N. Spencer and N. Rush, USDA, ARS, Pest Management Research Unit, Sidney, MT) and placed in cages (50 adults per cage; 2 cages total; metal cages were 30 cm × 30 cm × 30 cm and had fine mesh screens) in a greenhouse (16 h light:8 h dark) with each cage containing one knapweed plant. On a weekly basis, eggs were collected from the soil near the roots, brushed clean to remove soil and debris, disinfected (0.032% sodium hypochlorite for 4 min., followed by rapid rinses in sterile water), and, using sterile technique in a Biosafety Cabinet, placed in sterile petri dishes containing wetted filter paper that were then sealed with Parafilm. Egg dishes were maintained at 26 °C for 2–3 months and checked daily for hatch. The filter paper was periodically wetted with sterile water to ensure that proper moisture levels were maintained. To calculate days to hatch, the mean day for collection was determined and used as the ovipositional day. Therefore, these values have an inherent error of ±3.5 days. Percent hatch was determined only from the viable-appearing eggs (blond-colored and intact); damaged eggs (i.e., collapsed or darkened) were excluded from these calculations and averaged 31 ± 12% (SD) of the total eggs collected over the three generations. Egg damage may have been due to desiccation (although plants were watered twice daily) or the presence of nematodes/mites that were observed on occasion in the soil (the primary means of control for these pests was the use of presterilized soil). Artificial diets were prepared and poured into trays containing 32 cells (as described below). Prior to placement of the larvae onto the diet, one small hole (3–4 mm in depth) was bored into the center of the diet in each cell. Upon hatching, first instars were placed directly into this hole with fine forceps or a fine brush (using sterile technique as described above). This hole assisted the larvae in their ability to burrow into the diet. All larvae that hatched were used in the studies described in Tables 2A, 2B, and 3, or in the dormancy study described below. Larvae were maintained at 26 °C, 40–50% RH, 16 h light: 8 h dark. Diet cells were covered by a lightweight, darkened covering to restrict the amount of light they received. These cells were checked daily for adult emergence and "days to emerge" were defined as the number of days from hatch date to emergence date. Upon emergence, adults were placed into individual 0.8-liter round plastic containers (to track insects prior to weighing). The containers were fitted with filter paper, rolled shade cloth (for adults to hide in), a small petri dish (60 × 15 mm) containing a knapweed leaf (collected from greenhouse-grown plants) and a water wick (to keep the leaves fresh, wicks were wetted as needed), and covered with cheesecloth (200 mm × 200 mm) and a lid vented with a 22-mm hole. Adults were weighed 3 days later (the time needed for the adults to be able to firmly grasp plant leaves or stems). Approximately 50-60 adults, that were at least 3 days post-emergence, were placed into cages in the greenhouse as described above. One or two cages were utilized for each diet per generation, depending upon adult yield. Once the sexes could be distinguished morphologically, similar sex ratios in the cages were maintained between treatments at any given time.

For the short-term storage experiment, diet cells containing one larva each (average age of 6 days post-hatching) were placed into an incubator having a temperature that was gradually reduced from 26 to 10 °C (0.57 °C /day) and a light period reduced from 16 to 10 h over 4 weeks (with relative humidity remaining constant at 40–50%). These conditions remained stable for 4 weeks, followed by another 4-week period during which the temperature and light period were gradually raised back to the original levels. To help maintain proper humidity in the diet cells, cells were placed into plastic, ventilated containers lined with wetted filter paper that was rewetted daily. In this study, 64 larval diet cells containing standard diet (STD) and 33 cells containing standard diet with knapweed roots and leaves added (KWRL) were monitored.

Table 1
Standard meridic diet ingredients

Ingredient[a]	Amount	Source or manufacturer
Aeromycin	4 g	West Plains Vet. Supply, Springfield, MO
Agar	95 g	Moorehead, VanNuys, CA
Alpha-Cel	25 g	International Fiber, North Tonawanda, NY
Casein	126 g	National Casein, Chicago, IL
Formaldehyde (40%)	3 ml	Fisher Sci., St. Louis, MO
Linseed Oil (Raw)	26 ml	Bio-Serv, Frenchtown, NJ
Methyl-*p*-hydroxybenzoate	5.4 g	MP Biomedicals, Irvine, CA
Potassium sorbate	4 g	Pfizer, Hoffman Estates, IL
Propyl-gallate	0.8 g	Sigma Chem., St. Louis, MO
Soy protein	100 g	Ralston Purina, St. Louis, MO
Sucrose	135 g	General Stores, Univ. MO, Columbia, MO
Wesson's Salt Mix	36 g	Purina Mills Test Diet, Richmond, IN
Wheat Germ (Raw)	175 g	Atkins Natural Foods, Tulsa, OK
USDA Vitamin Mix #26862	36 g	Roche Vitamins, Fresno, CA

[a] These ingredients were added to 1000 ml water in the order specified in Section 2.

2.2. Artificial diet

The diet used was a modification of an artificial diet developed by N.R. Spencer and P.K. Peters at the BCIRL that was based on previously published formulations of other lepidopteran diets (Berger, 1963; Ignoffo, 1965; Vanderzant et al., 1962) (Table 1). The primary modifications made to the Spencer & Peters diet were the additions of soy protein and the deletion of potassium hydroxide. The latter component is normally incorporated into lepidopteran diets to elevate the pH to reflect the more basic environment of lepidopteran midguts. By contrast, coleopteran, including curculionid, larval digestive tracts have lower pH values than those of lepidopteran larvae (e.g., Hernández et al., 2003), therefore, we chose to formulate a less basic diet. To formulate the diet[3] (referred to here as "standard diet" or STD), water was brought to a boil and the agar, sucrose, and wheat germ were then added with continuous stirring. Wesson's salt mix and Alphacel were added after the diet temperature decreased to 85 °C. Vitamin mix, soy protein, and knapweed tissues (when appropriate) were added once the diet temperature had decreased to 70 °C or lower. Prior to inclusion into diet, knapweed leaves (3% by weight) and/or roots (1% by weight) were washed in 10% bleach (5.25% sodium hypochlorite) for 5 or 10 min, respectively, and rinsed in running water for 10 min. The tissues were then finely chopped in an electric food grinder (Mini-Mate, Cuisinart, East Windsor, NJ) and mixed into the diet using a food processor (Braun, Boston, MA). Diets were poured into plastic disposable rearing trays (each containing 32 cells) and sealed with the provided plastic adhesive lids (Bio-RT-32, C-D International, Pitman, NJ). Knapweed diets included either roots and leaves [KWRL] (first generation larvae) or roots only [KWR] (second and third generation larvae). All diets were stored at 4 °C until use.

[3] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

2.3. External morphology

Cyphocleonus achates adults were collected from cages in the greenhouse and external examinations were made to differentiate between the male and female. External morphologies were identified and images were generated using a stereomicroscope with an attached digital camera (Olympus SZX 12 with a DP10 camera or Leica MZ16 in conjunction with AutoMontage 5.0 software). In these studies, external characteristics were noted for each insect followed by internal examinations to confirm the sex. The characteristics compared between sexes were: antennae, tarsi, external markings, body length, weight, and abdominal shape. The differences in abdominal shape between the sexes were quantified by measuring the length (anterior–posterior direction) and the width of the last abdominal sternites using a miniscale measuring device (5 mm range with 0.1 mm divisions; BioQuip Products, Rancho Dominguez, CA) (see Fig. 1B).

2.4. Statistical analysis

NCSS Statistical Software (Kaysville, UT, USA) was used to determine diet and/or generational effects on *C. achates* development (Tables 2A and 2B) and differences in adult abdominal measurements between the sexes (Table 4). The former analyses had unequal replications. All analyses considered individual insects or egg dishes as one replication. Normality tests (for skewness and kurtosis) and the modified Levene equal variance tests were performed on all data sets (Tables 2A, 2B, and 4). For data that passed these tests, analysis of variance (ANOVA) was performed in conjunction with Scheffe's multiple-comparison test. Kruskal–Wallis multiple-comparison Z value tests in combination with the Bonferroni test (in order to adjust the Z value for multiple tests) were performed for data sets having non-normal distributions and equal variances between treatments. For data sets with unequal variances, two-sample t tests were performed followed by the Kolmogorov–Smirnov test for different distributions.

3. Results

3.1. Evaluation of insect growth, development, and reproduction

We reared *C. achates* larvae for three generations on a meridic diet, with or without plant tissue (Tables 2A, 2B, and 3). For the first generation, the knapweed supplemented

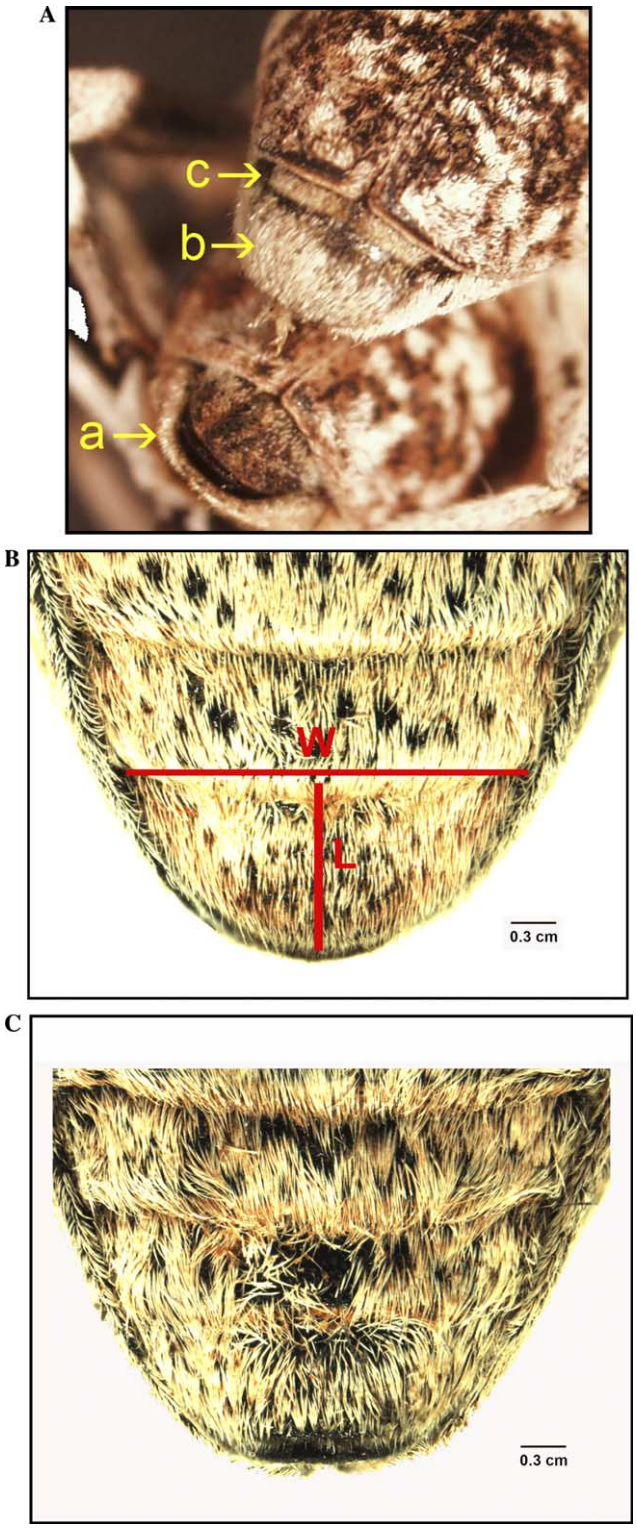

Fig. 1. Images of *C. achates* showing external characteristics used to sex the adults. (A) Caudal view of mating pair (10×). Arrows indicate: (a) cloacal opening and tapered terminal abdominal segment of female; (b) rounded male terminal abdominal segment pointing ventrally; and (c) sclerotized penultimate abdominal tergite of male. (B) Ventral view of adult female abdominal segment. Note, red lines accompanied by the following letters indicate: W, width and L, length. (C) Ventral view of adult male terminal abdominal segment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

diets contained roots and leaves, whereas for later generations only roots were added. Knapweed leaves were initially added to determine if they would enhance diet performance. No differences were seen between diets with leaves only, roots only, or leaves and roots in a study that ended while first generation results of the present study were being collected (data not shown). Therefore, leaves were excluded from the diet in the later generations, especially as *C. achates* larvae are primarily root feeders (Stinson et al., 1994) and leaves generate leaf-specific allelochemicals that can be toxic (e.g., Locken and Kelsey, 1987).

The addition of knapweed tissues to the diet did not significantly influence larval growth, development, or adult reproduction. In the egg stage, the parameters measured were percent hatch (ranging from 42.9 to 59.1%) and time to hatch (20.0–23.2 days) (Tables 2A and 2B) (Note: the mean number of eggs per dish was 21.7 for generation 1; 80.5 and 115.7 for STD and KWRL diets for generation 2, respectively; and, 33.0 and 39.8 for STD and KWR diets in generation 3, respectively). In the adult stage, days to emergence (52.0–54.1 days) and day 3 post-emergence (Tables 2A and 2B) were monitored. Within a given generation, there were no significant differences when comparing the insects reared on standard (STD) and knapweed (KWRL or KWR) diets. When comparing generations, there were slight differences which were significant for two parameters. The days to emergence were significantly fewer for the second generation insects (with egg hatch beginning in January and emergence beginning in March) as compared to the KWRL-diet fed first generation insects (with egg hatch beginning in December and emergence beginning in February) and the third generation insects (with egg hatch beginning in June and emergence beginning in August). The probability levels for these comparisons were as follows (gen. = generation): 0.9599 (second gen. STD = KWR), 0.0144 (first gen. KWRL > second gen. STD), 0.0059 (third gen. STD > second gen. STD), 0.0092 (third gen. KWR > second gen. STD), 0.0032 (first gen. KWRL < second gen. KWR), 0.0020 (third gen. STD > second gen. KWR), and 0.0009 (third gen. KWR > second gen. KWR). These differences in days to emergence represent only a ~1–3% difference, suggesting the biological significance may be minor. Additionally, the adult weights of the larvae fed standard diets in the second generation were significantly less (by approx. 13%) than those fed knapweed diets in the third generation (101.9 mg vs. 117.0 mg; Z value = 4.5471).

The primary stages where mortality occurred were identified for each treatment within a generation (Table 3). Statistical analysis could not be performed on these values as only one data set was generated for each generation. The highest percent mortality occurred when the insects were still within the diet, i.e., the larval and pupal stages (ranging from 38.3 to 49.6%). Within the first and second generation, there were similar levels of insects dying as larvae or pupae for both diets. By contrast, the last generation had a higher proportion of insects dying in the diet cells for those fed KWR diets (49.8%) than those

Table 2A
Statistical analysis of growth and development data

		% Hatch	Days to hatch	Days to emerge	Adult weight
Normality—Skewness	Test value	−1.1724	20.7053	3.9632	36.3058
	Probability level	0.2411	0.0000	0.0001	0.0000
	Accept/reject	Accept	Reject	Reject	Reject
Normality—Kurtosis	Test value	−1.7408	14.4314	3.6186	22.4520
	Probability level	0.0817	0.0000	0.0003	0.0000
	Accept/reject	Accept	Reject	Reject	Reject
ML equal variance	Test value	1.8213	5.6049	4.4232	0.4757
	Probability level	0.0776	0.0002	0.0046	0.8526
	Accept/reject	Accept	Reject	Reject	Accept
Test performed[a]		Scheffe's MCT	Two-sample *T* test with K–S DD Test	Two-sample *T* test with K–S DD Test	Kruskal–Wallis MCT with Bonferroni Test
		$\alpha = 0.050$	$\alpha = 0.050$ (2-sided), 0.025 (1-sided)	$\alpha = 0.050$ (2-sided), 0.025 (1-sided)	$\alpha = 0.050$
		DF = 144			*Z* values > 3.1237 (for significance)
		MSE = 544.219	DMN range = 0.0979–0.3862	DMN range = 0.0415–0.3932	
		CV = 4.003	Prob. level range = 0.0000–0.0814	Prob. level range = 0.0009–0.9599	

[a] *Abbreviations:* ML, modified Levene equal variance test; MCT, multiple choice comparison test; MSE, mean square error; CV, critical value; K–S DD test, Kolmogorov–Smirnov test for different distributions; DMN, maximum difference between the two empirical distribution functions (i.e., the Kolmogorov–Smirnov test statistic); Prob. level, probability level.

Table 2B
Growth and development of *C. achates* on different larval diets over three generations[a]

Generation[b]	% Hatch (*N*)	Days to hatch (*N*)	Larval diet	Days to emerge (*N*)	Adult weight (*N*)[c] (mg)
1	52.5 ± 6.0 (28) a	20.0 ± 0.4 (299) a	STD	52.0 ± 1.2 (75) ab	111.7 ± 3.8 (70) ab
			KWRL	54.1 ± 1.0 (65) b	108.4 ± 2.9 (61) ab
2	54.5 ± 6.9 (10) a	22.8 ± 0.3 (451) a	STD	52.6 ± 0.5 (260) a	101.9 ± 1.5 (241) a
	59.1 ± 6.8 (10) a	23.2 ± 0.2 (510) a	KWR	52.4 ± 0.4 (299) a	106.6 ± 1.5 (288) ab
3	42.9 ± 5.1 (18) a	22.0 ± 0.4 (291) a	STD	53.7 ± 0.4 (167) b	107.2 ± 1.6 (166) ab
	46.0 ± 4.1 (21) a	21.1 ± 0.3 (368) a	KWR	53.1 ± 0.5 (175) b	117.0 ± 4.9 (170) b

[a] Values represent means ± SEM with the number (*N*) of egg dishes (Column 2) or individual insects (Columns 3, 5, and 6) being recorded in parenthesis. Each *N* was considered one replication. Values having different letters within each column represent statistically significant differences (see Section 2 for specific tests performed).

[b] In the first generation, eggs were oviposited by adults from the field. The resulting larvae were set up on either STD or KWRL diet. In the second generation, neonates from adults of larvae fed STD diet were placed onto STD diet and those from adults of larvae fed KWRL diet were placed onto KWR diet. In the third generation, neonates from adults of larvae fed STD diet were placed onto STD diet and those from adults of larvae fed KWR diet were placed onto KWR diet.

[c] Adult weights were recorded on the third day post-emergence. The variation in *N* between the "Days to emerge" and "Adult weight" is due to fewer healthy-appearing adults being weighed than had actually emerged.

fed STD diets (38.3%). A subset of diet cells from each generation was dissected and the percentage of dead larvae vs. pupae was determined. The majority of insects died in the larval stage rather than the pupal stage. Of the cells examined, the percentage that died as larvae (vs. pupae) in each generation is as follows: 81.3% STD-fed and 71.3% KWRL-fed (first generation); 91.9% STD-fed and 96.6% KWR-fed (second generation); and 88.1% STD-fed and 96.7% KWR-fed (third generation). Of the dead insects found in the cells over the three generations, many were first and early second instar larvae (with a mean of 52.4 ± 7.7% SEM over three generations) based on approx. head-capsule size (*C. achates* is known to have four instars, Stinson et al., 1994).

The percentage of the insects that emerged as healthy-appearing adults (those with no significant deformations) were also noted (Table 3, last column). Within-generation values for percentage of viable adults were quite close for the first and second generations. For the third generation, these values ranged from 48.3% (for KWR) to 57.6% (for STD). As with the other Table 3 data, statistical analysis could not be performed on these numbers due to only one data set per generation. Nevertheless, the most consistent results over the three generations were seen in insects fed the standard diet devoid of knapweed tissues (STD). The greater inconsistency of the knapweed tissue-containing diets may be due to variations in plant quality, either before tissue preparation (e.g., plant age or response to the environment) or after tissue addition to the diet (e.g., preservation issues once in the diet). It should also be noted that few grossly deformed adults were observed at any time, with the percentage of larvae emerging as deformed adults ranging from 1.9 to 6.5%. These deformities were primarily in the elytra or underlying wings (e.g., split elytra, bent wings) and may have been caused by a slight drying of the diet cells.

Table 3
Percent outcomes in each generation of *C. achates* fed artificial diets[a]

Generation	Larval diet	% Larval or pupal mortality[b]	% Deformed adults[c]	% Adult mortality[d]	% Viable adults
1	STD	48.0	2.0	1.3	50.7
	KWRL	49.6	5.2	2.2	48.2
2	STD	39.6	5.8	2.7	57.7
	KWR	39.6	6.5	1.8	58.6
3	STD	38.3	5.5	4.1	57.6
	KWR	49.8	1.9	1.9	48.3

[a] The following number of larval diet cells were set up for each generation: 148 (SF) and 135 (KWRL) in the first generation; 451 (SF) and 510 (KWR) in the second generation; 290 (SF) and 362 (KWR) in the third generation.
[b] Pupal mortality was generally accompanied by deformities.
[c] Deformed adults were not placed in mating cages.
[d] Indicates adults that died within 3 days after emergence.

A study was undertaken using a subset of the first generation to determine if the *C. achates* could survive (i.e., be stored) in the diet cells for a longer period of time at lower temperatures and shorter light periods. A marked decrease in emergent adults resulted as compared with standard conditions, with only 35.6% STD-fed insect (of 64 total) and 19.4% KWRL-fed insects (of 33 total) emerging as healthy-appearing adults. The percentage of deformed adults that died within 3 days after emergence was higher for the insects reared in the slow-growth conditions than those in the standard conditions. For insects reared in the slow-growth conditions, 10.2% of the adults were deformed and 8.5% died shortly after emergence for the STD diet-fed insects. For the KWRL diet-fed insects, 6.5% of the adults were deformed and 12.9% died shortly after emergence (compare with Table 3 results, first generation). The weights of the adults in this study were also generally lower than those reared under standard conditions, with the STD diet-fed adults weighing 91.8 ± 6.0 mg (statistically lower than the mean weight of the third generation KWR diet-fed insects) and the KWRL diet-fed adults weighing 82.6 ± 11.0 mg in the slow-growth conditions (mean ± SEM). Nevertheless, adults were still reproductively active, producing viable eggs (with a hatch rate of 26–28% for both diets). A higher percentage of insects in this study died as pupae vs. larvae as compared to those reared under standard conditions, with 45.6% dying as pupae for STD diet-fed and 44.4% dying as pupae for KWRL diet-fed insects (compare with above results).

3.2. External morphology

Differences between male and female adult *C. achates* can be difficult to determine as this species does not have obvious sex-related markings. Of the characteristics examined, the only reliable diagnostic character was abdominal shape (Fig. 1). Female abdomens are caudally tapered (pointed) with cloacal openings facing outwards. Conversely, male abdomens have terminal segments that are deflexed ventrally, causing their cloacal opening to face ventrally. These differences in abdominal shape were quantified by measuring the length and width of male and female terminal sternites (Table 4, Figs. 1B and C). There were significant differences in the length and the length/width ratios of the sternites when comparing males and females, with no significant differences being noted for the sternite widths. Thus, the longer terminal sternites create a more tapered appearance for the females as compared with the males. Abdomens of males and females can also be distinguished by their sclerotization (with the last two male tergites being sclerotized, but only the terminal tergites being sclerotized in the female) and segment numbers (males have eight abdominal segments and females seven segments). To best observe most characteristics just mentioned for determining the sex of an individual, the abdomen must be partially or fully extended. This abdominal extension is generally most common in reproductively active adults. A typical mating pair will clearly show most of the characteristics important for sexing *C. achates* adults: the caudally pointing cloacal opening in the female; the semi-circular male terminal segment pointing ventrally; the scleritized penultimate abdominal tergite of the male (Fig. 1A).

Table 4
Differences in the sizes of the terminal adult sclerites[a]

	Length (mm)[b]	Width (mm)[b]	Length/width
Female	1.01 ± 0.013 b	2.34 ± 0.024 a	0.43 ± 0.005 b
Male	0.93 ± 0.028 a	2.28 ± 0.064 a	0.41 ± 0.009 a

[a] Values represent means ± SEM ($N = 10$), with each N being considered one replication. Significant differences within columns are indicated by differing letters. Statistical tests performed (see Tables 2A and 2B for abbreviations): Kruskal–Wallis MCT with Bonferroni Test, Z value > 1.9600 (length); two-sample T test with K–S DD Test, $\alpha = 0.05$ (2-sided), $\alpha = 0.025$ (1-sided), DMN = 0.2–0.3, Prob. level = 0.7869 (width); Scheffe's MCT, $\alpha = 0.05$, DF = 18, MSE = 0.00047, CV = 2.1009 (length/width).
[b] See Fig. 1 for clarification of length and width measurements.

4. Discussion

In the field, *C. achates* produces only one generation a year, with adults emerging over a 2- to 3-month period (Clark et al., 2001; Stinson et al., 1994; Story et al., 1997; Wikeem and Powell, 1999). Therefore, if one were to depend on natural populations of *C. achates* for the collection of adults to use to control spotted knapweed, there is only a narrow window of time within any location to make these collections. With the combination of artificial diets for larvae and knapweed plants as ovipositional substrates and food for adults, we obtained adults year round with overlapping generations from October, 2001 to December, 2002 (at which time the experiment was terminated). We found slight or no differences in insect quality between generations and larval diets in the observed parameters, including: percent egg hatch, time to egg hatch, time to adult emergence, day 3 adult weights, percent adult emergence,

and percent mortality/deformity in the different stages. Furthermore, a preliminary experiment performed while the diet was still under development resulted in rearing *C. achates* through four generations with few differences within and between generations (data not shown). Taken together, these data indicate that uniform results can be generated throughout the year using our meridic diet in the larval stage.

There are some differences in the egg-laying behavior and the length of time to egg hatch between our studies and those of Stinson et al. (1994). In the latter study, last instar larvae or pupae were collected from field populations in eastern Austria, central Hungry, and eastern Romania. The resulting adults from these insects oviposited in the root crown (similar to field observations) and egg hatch took place in 10–12 days (when incubated at 25 °C). In our studies, eggs were oviposited in the soil near, but not in, the root or root crown and eggs hatched in 20–23 days (when incubated at 26 °C). The differences between the ovipositional results may be due to soil conditions. In the field, knapweed patches are often located in dry and/or sandy soil (Sheley et al., 1998) where oviposition into the roots is necessary to ensure that appropriate moisture levels are maintained. In our study, plants were watered one to two times each day (depending upon the season), allowing the eggs to readily survive when deposited in the soil. By contrast, it is unclear from Stinson et al. (1994), what moisture levels were maintained in the soil. Differences in time to hatch between studies could not be due to differences in incubation temperatures nor in methodology (both of which were nearly identical). Differences in hatching time may be due to the adaptations the *C. achates* population (from which we obtained our eggs) underwent during establishment from Eurasia to Montana or unexplained differences in the adults because ours were reared on artificial diets.

A high percentage of the insects that died prior to adult emergence died as larvae, many of which were early instars. These deaths could be due to either insect handling or diet formulation issues. If the latter, a lack of specific micronutrients or phagostimulants or the addition of deleterious components or feeding deterrents could have caused early mortality. The larval deaths could also be due to a preprogrammed delay in development that slowed down or stopped larval growth and, therefore, resulted in the diets drying out and/or aging before insect development was completed. Some insect species, including weevils, vary the duration of their progeny's life cycle to ensure success of at least a portion of their progeny (Danks, 1992) through either the onset of dormancy or a decrease in the development rate. The chestnut weevil, *Curculio elephas* Gyllenhal, is thought to employ a bet-hedging strategy for progeny of the same cohort for these purposes (Menu and Desouhant, 2002). Using a somewhat different strategy, *Hylobius transversovittatus* Goeze adults produce progeny that develop over a 1- or 2-year period depending upon when in the season their eggs are oviposited, with those oviposited later taking longer to develop (Blossey, 1993). Therefore, the early mortality of *C. achates* larvae may have been due to a number of factors, including diet-related deficiencies or the preprogramming of the progeny for slower or delayed developmental rates that resulted in incomplete development due to diet aging.

There are numerous reports describing the development of artificial diets for selected weevils, some of which, like *C. achates*, are root feeders as larvae. Many of these artificial diets utilized varying quantities of the host plant in order to obtain successful outcomes. A diet developed for the root-boring weevil, *Hylobius transverovitatus*, contains ~24% ground purple loosestrife roots (by weight), with a version of this diet being modified for *C. achates* by the addition of ground knapweed root in place of purple loosestrife (Blossey et al., 2000; Blossey and Eberts, 2001). Similarly, a diet generated for the root- and leaf-feeding weevil of sugar beet, *Aubeonymus mariaefranciscae* (Roudier), contains ~19% sugar beet root homogenate (volume to weight) (Farinos et al., 1999). Diets developed for the white pine weevil, *Pissodes strobi* (Peck), have from approx. 2 to 10% ground pine bark (by weight) (Trudel et al., 1994; Zerillo and Odell, 1972). Pine root collar weevil, *Hylobias radicsi* Buchanan, and pales weevil, *Hylobias pales* (Herbst), diets contain approx. 1–27% ground red pine phloem (Hunt et al., 1992; Thomas, 1969), although Clark (1972) replaced the phloem with β-sitosterol and had positive results. A diet for the sweetpotato weevil, *Euscepes postfasciatus* (Fairmaire), contains ~8% (by weight) dried sweet potato powder or a comparable amount of a sweet potato flake foodstuff (Shimoji and Yamagishi, 2004) and that for the red palm weevil, *Rhynchophorus ferrugineus* Olivier, contains ~0.7% coconut fiber (Alarcón et al., 2002). Nevertheless, successes have been reported for curculionid diets devoid of host plants that feed on fruit or flowers as larvae, such as the plum curculio (*Conotrachelus nenupar* (Herbst)) (Yonce et al., 1970), and the boll weevil (*Anthonomus grandis grandis* Boheman) (Lindig, 1980). Therefore, ours is one of the first meridic diets that is free of host plant that has been developed for weevils of root-feeding larvae (with the exception of a diet recently developed for *C. achates* and *H. transversovittatus* by N. Tomic-Carruthers and C. McGuin, USDA, APHIS, PPQ, CPHST, Albany, CA, personal communication).

A study was conducted in order to determine if *C. achates* larvae could be stored for short time-periods under low temperature-short day conditions. This study was partly based on a Stinson et al. (1994) observation that *C. achates* overwinter in the field (in Eurasia) as larvae, primarily second instars with some overwintering as first instars. In our study, when young larvae (average of 6 days old; i.e., first instars, according to Stinson et al., 1994) were placed for 1 month under growth-retarding conditions, a lower percentage emerged as healthy adults as compared with those maintained under standard conditions. Additionally, these adults generally weighed less than those reared under standard conditions and a higher percentage died as pupae (usually deformed) and not as larvae, as compared to

insects reared under standard conditions. The higher pupal mortalities and deformities suggest the insects attempted development but were unable to complete it. These results may have been due to the drying (although precautions were taken to limit this process, see Section 2) or aging of the diet, or to suboptimal temperature or light conditions affecting dormancy onset, maintenance or termination. Therefore, to improve adult yield, alterations may need to be made to the diet or to growth conditions to ensure larval development is successfully completed. Nevertheless, we have shown that *C. achates* larvae can be stored for short time-periods at cooler temperatures with reduced lighting and still successfully emerge and lay viable eggs.

Distinguishing sexual traits is important for studies involving *C. achates* reproduction. We observed that the main distinguishing characteristics between males and females were the shape, orientation, number, and sclerotization of their terminal abdominal segments. Wikeem and Powell (1999) also used abdominal shape and cloacal openings when sexing *C. achates* but did not elaborate on their method. Other weevils have been sexed by focusing on features of the abdomen as well: the female of the white pine weevil (*P. strobi*) was differentiated from the male by a thinner, more curved seventh sternite with a more visible membranous lobe (in males, this sternite is thick and elevated with a harder to visualize lobe) (LaVallée et al., 1993); in West Indian sweetpotato weevils (*E. postfasciatus*), the posterior edge of the eighth tergite is swollen in males but sharpened and flat in females (best seen when submerging weevils in water at 20–25 °C for a few seconds) (Hiroyoshi et al., 1996); for the apple blossom weevil (*Anthonomus pomorum* L.), males are distinguished from females by a characteristically distinct division of the last two tergites and a smaller eighth tergite having a dense covering of setae (Duan et al., 1999); the boll weevil (*A. grandis*) is routinely sexed by the presence in males of a distinct notch in its eighth tergite (Sappington and Spurgeon, 2000); and, in the mango pulp weevil (*Sternochetus frigidus* (Fabr.)), males have truncated seventh and eighth tergites and the female has only seven tergites with the terminal tergite being larger, more tapered than the terminal male tergites (DeJesus et al., 2002). The sexing of *C. achates* adults is similar to that of *E. postfasciatus*, in that the behavior of the insects can assist in ascertaining their sex, *A. pomoroum*, in that the males have distinct divisions between their final two tergites, with both being sclerotized, and have a dense covering of setae on their last tergite (seen in some *C. achates* males), and, *S. frigidus*, in that the last female tergites are more tapered than those of the males. Therefore, the characteristics we found useful for distinguishing between the sexes are not markedly different from those of other curculionids.

In summary, the development of a meridic diet that allows for the continual, year-round production of *C. achates* will increase the availability of this weevil for release in different geographical areas throughout the United States at appropriate times for each region. Furthermore, this increase in availability will allow for the performance of studies aimed at understanding the interactions of *C. achates* with their environment and host plant—both of which will, in the long term, expand and maximize their use as biocontrol agents.

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